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A Research Note

Gamma Radiation Resistance of *Clostridium botulinum* 62A and *Bacillus subtilis* Spores in Honey

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ABSTRACT

Irradiation D values for the natural bacterial flora of two samples of raw (bulk) honey were 7.50 and 1.91 kGy; for two samples of retail honey the D values were 5.66 and 3.49 kGy. Irradiation D values of *Clostridium botulinum* 62A spores inoculated into three honey samples and into water were respectively, 8.11, 9.38, 12.77, and 2.07 kGy. Similar D values for *Bacillus subtilis* spores were 3.42, 3.35, 4.00, and 1.43 kGy. The radiation resistance of *C. botulinum* and *B. subtilis* spores in honey and in sugar syrups was a function of water content.

Honey is a microbiologically stable product owing to its low water content and its high concentration of sugars (*18*). Its sticky nature and its stability ensures that bacterial spores from dust or from the activities of the bees will readily adhere and will remain indefinitely. The presence of *Clostridium botulinum* spores in honey consumed by infants has been linked to some cases of the toxico-infectious disease, infant botulism (*1,3,10*). The spores of this organism also have been found in other honey samples; Sugiyama et al. (*16*) tested a total of 241 retail and producer honey samples and found that 18 contained spores of *C. botulinum,* the maximum number in any sample was 7 spores per 25-g sample. Huhtanen et al. (*11*) found six positive samples out of 80 tested. Kautter et al. (*13*) reported an incidence of two positive retail samples of 100 tested while Hauschild et al. (*10*) found a positive sample associated with a case of infant botulism but failed to find spores in 106 other samples. Others have not found spores in honey (*7,9*).

Thermal treatment has been tried as a means of reducing bacterial populations of honey. Calesnick and White (*4*) were able to substantially reduce populations of *Bacillus larvae* spores in honey by increasing its acidity with phosphoric acid, diluting it with water, and subjecting it to temperatures above 100°C. High velocity electrons were used by Katznelson et al. (*12*) for reducing the levels of *B. larvae* in honeycombs and honey. The purpose of this study was to determine the feasibility of using gamma radiation to eliminate *C. boulinum* spores from honey; the effect of irradiation on the normal flora of honey was also studied.

MATERIALS AND METHODS

Honey samples and sugar syrup

Honey samples were obtained from 55 gal drums of two honey packing companies or from retail sources. The composition of the sugar syrups was based on the analyses of White (*18*): syrup 1 contained 1.69% sucrose, 35.08% glucose, 42.83% fructose, and 20.34% H_2O; syrup 2 contained 1.82% sucrose, 37.64% glucose, 46.10% fructose, and 14.54% H_2O. The syrups were made by dissolving the ingredients in the water at a temperature of 70°C, with stirring, until solution was complete. The two syrups represented the extreme variations in water content of honey as reported by White (*18*).

Cultures

Cultures of *Bacillus subtilis*, NRRC 744 and *Clostridium botulinum* 62A (NRCC B1218) were from the Northern Regional Research Center, U. S. Department of Agriculture, Peoria, IL. A spore suspension of *B. subtilis* was made from plate count agar slants incubated 4 d at 35°C. The growth was washed off with water, centrifuged at 3000 x g for 15 min, resuspended in water and heated in a water bath for 10 min at 80°C. Spores of *C. boulinum* were prepared from tryptic soy broth cultures incubated in an anaerobic jar (BBL, Cockeysville, MD) for 10 d at 35°C. The cultures were centrifuged at 3000 x g for 15 min, resuspended in water, and heated at 80°C for 10 min. Spore counts of *B. subtilis* were made in plate count agar incubated 2 d at 35°C. Spore counts of *C. botulinum* were made in Brewer anaerobic agar plates incubated in an anaerobic jar (BBL) for 3 d at 35°C. The natural aerobic bacterial flora of honey was determined in plate count agar incubated at 30°C for 2 d. For the irradiation experiments, two portions of 20 g honey were placed into 208 x 107 aluminum cans which were sealed under N_2. One set was kept as a control; the other was irradiated. The inoculum was 0.1 ml of the appropriate spore suspension.

Irradiation

Cans of honey were irradiated at 0 to 4°C (in ice water) with ^{137}Cs source emitting 125 Gy/min. Irradiation D values were calculated from the function:

$$D = \text{dose (kGy)} / (\log CFU_a - \log CFU_b)$$

where a was the count before and b the count after irradiation.

Standard deviations were calculated from triplicate plate counts.

RESULTS

The effect of irradiation on the indigenous microflora of several honey samples was the object of a preliminary study. Two samples each of bulk and retail honey irradiated at a dose of 3.0 kGy showed reductions in log CFU/g from 2.28, 4.78, 2.39, and 2.94 before irradiation to 1.88, 3.21, 1.86, and 2.08, respectively, giving D values (in kGy) of 7.50, 1.91, 5.66, and 3.49, respectively. The D values of the normal flora of other honey samples ranged from 3.49 to 7.50 kGy, values which equal or exceed those reported by Grecz et al. (*8*) for the most resistant spores of *C. botulinum* (irradiated in buffer); these were 0.34 Mrad (3.4 kGy) for strain 33 type A *C. botulinum* and 0.33 Mrad (3.3 kGy) for strain 53 type B.

The effect of irradiation on spores in two retail honey samples is shown in Table 1. The D values for *C. botulinum* spores were 9.38 and 12.77 kGy for the two samples while the D values for *B. subtilis* spores were 3.35 and 4.00. The D values in water, at an irradiation dose of 6.0 kGy, could not be calculated since there were no irradiation survivors.

Irradiation D values for spores of *C. botulinum* and *B. subtilis* irradiated in diluted or nondiluted honey or in sugar syrups are shown in Table 2. *C. botulinum* spores in water irradiated at 3.0 kGy had a D value of 2.07 while in undiluted honey it was 8.11; in honey diluted 1:1 or 1:7 the D values were 3.06 and 3.37, respectively. In sugar syrup 1 with a water content of 20.34%, the D value was 3.45 and in sugar syrup 2 with a water content of 14.5%, the D value was 20.7. Spores of *B. subtilis* were more sensitive to irradiation; the D value in water was 1.34, in undiluted honey it was 3.42, while in honey diluted 1:1 or 1:7 the D values were 2.45 and 1.85. In sugar syrup the D values were 3.90 for the syrup with 20.34% H_2O and 4.05 for the syrup with 14.54% H_2O.

TABLE 1. *Effect of irradiation on spores of* Clostridium botulinum *62A and* Bacillus subtilis *in honey*[a].

Spores[b]	Medium	Log CFU/g Irradiaiton dose None	SD	6 kGy	SD	D value (kGy)
C. botulinum	H_2O	4.73	1.1	<1[c]	-	<1.61
" "	Honey A	4.72	0.9	4.08	0.8	9.38
" "	Honey B	4.73	0.6	4.26	1.6	12.77
B. subtilis	H_2O	4.23	1.4	<1[c]	-	<1.86
" "	Honey A	4.28	1.1	2.49	1.2	3.35
" "	Honey B	4.23	1.6	2.73	1.3	4.00

[a]The honey samples were retail and were selected for their low bacteria counts (no colonies in 1 ml of 1/10 dilution on plate count agar); sample A was light honey, B was dark.

[b]Spores were added to 10 g of honey (0.1 ml of approximately 10^6/ml suspensions).

[c]There were no colonies from 1 ml of 1/10 dilution, *B. subtilis* counts made on plate count agar; *C. botulinum* counts were made in Brewer anaerobic agar incubated under anaerobic conditions.

DISCUSSION

Ionizing radiation exerts its antimicrobial effect [e.g., on DNA, Grecz et al. (*8*)] either by direct collision of high energy photons with sensitive cellular constituents or indirectly by the formation of hydrogen or hydroxyl free radicals from water molecules which then act on molecules and atoms in the cell (*14*). Thus, the paucity of water molecules in honey probably accounts for the resistance of the bacterial spores to gamma radiation, although it is possible that honey also has radio-protective free radical scavengers such as ascorbic acid, fumarate or glutamate (*15*). The results of the present study indicate that irradiation can be used to reduce the number of spores of *C. botulinum* or *B. subtilis* in honey, but the process was more efficient when honey was diluted 1:1 or 1:7. This effect of water content was substantiated by the results obtained from the two sugar syrups; the one with the smallest water content was considerably more resistant to irradiation.

Completely dry materials can be sterilized by ionizing radiation. Dehydrated cultures of enterococci were sterilized by Christensen and Sehested (*5*) in a study of the efficacy of radiation for sterilization of medical supplies; Tjaberg et al. (*17*) found that spices could be sterilized with a dose of 1.58 Mrad (15.8 kGy). In order to reduce the normal bacterial levels of the bulk honey samples, used in this study, to nondetectable levels (for example, to less than 10 CFU per g) with gamma radiation would require doses of 9.6, 7.2, 8.2 and 6.7 kGy, respectively. On the other hand, in order to reduce a theoretical population of log 4.73 *C. botulinum* spores, with a radiation D value of 12.77 kGy (Table 1) to the same level, a dose of 47.63 kGy (4.76 Mrad) would be needed. However, these levels are unlikely in honey; Sugiyama et al. (*16*) reported that the highest level in 241 samples tested was 7 spores in 25 g.

Cleland and Pageau (*6*) made a comparison of the expense and utility of using energetic electrons from linear accelerators versus photons from gamma radiation sources. The limited range of electron beams precludes their use for sterilization of dense materials though plastic medical devices are routinely sterilized with them. Gamma rays with their greater penetrating power make their use a more feasible alternative for reducing bacterial populations in a viscous material such as honey.

The study reported here did not address the problem of possible organoleptic changes in irradiated honey, but there were no obvious color or flavor differences between the irradiated and the control samples.

Gamma radiation could be used for reducing the levels of *C. botulinum* spores in honey. The incidence of infant botulism in the United States averages about 42 cases per year and honey was consumed by about 33% of the af-

TABLE 2. *Effect of diluted honey and sugar syrups on gamma radiation sensitivity of* Clostridium botulinum *62A and* Bacillus subtilis *spores.*

Spores	Medium	Dose (kGy)	H_2O (%w/w)	Log CFU/g Control	SD	Irradiated	SD	D value (kGy)
C. botulinum	H_2O	3.0	100	4.23	0.85	2.78	1.25	2.07
	Honey[a]	6.0	16	4.35	1.12	3.61	2.26	8.11
	"	6.0	58	4.31	0.69	2.35	0.55	3.06
	"	3.0	90	4.18	0.15	3.29	1.00	3.37
	Syrup 1[b]	6.0	20.3	4.24	0.55	2.50	2.30	3.45
	Syrup 2	6.0	14.5	4.27	0.96	3.98	1.32	20.7
B. subtilis	H_2O	3.0	100	4.91	1.08	2.67	0.61	1.34
	Honey	6.0	16	4.91	0.69	3.16	1.33	3.42
	"	6.0	58	4.77	1.65	2.32	0.96	2.45
	"	3.0	90	4.87	1.21	3.22	0.15	1.85
	Syrup 1	6.0	20.3	4.83	0.63	3.29	1.15	3.90
	Syrup 2	6.0	14.5	4.97	0.55	3.49	1.59	4.05

[a]Honey of higher water content obtained by diluting 1:1 or 1:7.
[b]Sugar syrup 1 contained (w/w) 1.69% sucrose, 35.08% glucose, 42.83% fructose, and 20.34% H_2O. Sugar syrup 2 contained 1.82% sucrose, 37.64% glucose, 46.10% fructose, and 14.5% H_2O.

fected infants (*2*). Irradiating with a 2D (16 kGy or 1.6 Mrad) dose would result in a reduction of 14 cases of infant botulism per year if it is assumed that every sample of honey consumed by the infants contained a spore capable of germinating, multiplying in the intestinal tract of the infant, and producing enough toxin to cause disease.

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